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Via email to: niceatm@niehs.nih.gov

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Re: Federal Register Vol. 70, No. 238, pp 74533-4, December 12, 2006: NTP Interagency Center for the Evaluation of Alternative Toxicological Methods; Announcement of an Independent Scientific Peer Review Meeting on the Use of *In Vitro* Pyrogenicity Testing Methods; Request for Comments

Dear Dr. Stokes:

These comments are submitted on behalf of the more than 10 million U.S. members of the Physicians Committee for Responsible Medicine, People for the Ethical Treatment of Animals, the Humane Society of the United States, the Alternatives Research & Development Foundation, the American Anti-Vivisection Society, and the Doris Day Animal League. We appreciate the opportunity to review ICCVAM's recommendations for five *in vitro* pyrogenicity tests (IVPTs) conducted using either human whole blood or human monocytic cell lines, and to provide comments regarding ICCVAM's "Draft Test Method Recommendations" (Recommendations) and "Draft Background Review Document" (BRD) on these methods. These comments incorporate by reference an earlier submission dated January 17, 2006.

At the outset, it should be stated that the parties to this submission have always endeavored to regard ICCVAM and its member agencies as federal partners who share our commitment to reducing, refining, and ultimately replacing the use of animals in regulatory toxicology. However, the abbreviated number of methods reviewed by ICCVAM and accepted by federal agencies in recent years raises concern over the genuine commitment to progress in the 3Rs by some federal agencies and/or their representatives on ICCVAM. The pyrogenicity BRD and Recommendations currently under discussion represent a glaring case in point.

ICCVAM's Recommendations accept the use of IVPTs only for the detection of lipopolysaccharide-mediated (LPS) pyrogenicity induced by gram-negative bacterial endotoxins "in materials currently tested in the RPT" (rabbit pyrogen test). Thus, for practical purposes, ICCVAM's Recommendations do not support the use or regulatory acceptance of these methods for the detection of gram-positive bacterial, fungal, or viral pyrogens. Moreover, ICCVAM specifically states that it does not regard the IVPTs as full replacements for the Limulus amebocyte lysate (LAL). Its Recommendations further state that in order to be considered as potential replacements for the RPT for the detection of non-LPS-mediated pyrogenicity, "additional studies that include a broader range of pyrogenic materials are recommended…such studies should include parallel RPT testing." More specifically, "when a positive non-endotoxin-mediated RPT result is encountered, this same sample should be subsequently tested *in vitro*."[1]

Despite the extensive discussion of the 3Rs throughout the BRD and Recommendations, it is not clear if or how ICCVAM's Recommendations could contribute to a meaningful reduction in animal use in

pyrogenicity testing if in fact we are not looking to replace the BET and continued comparisons to—and confirmatory testing in—the RPT are required for these methods.

We therefore strongly urge ICCVAM to significantly revise its Recommendations and BRD to more accurately reflect the potential use of these methods as full replacements for both the LAL and RPT. The available evidence shows that the IVPTs are fully valid for the detection of all pyrogens. We also strongly encourage ICCVAM to delete the recommendation regarding the conduct of de novo RPTs to further demonstrate *in vivo/in vitro* concordance.

General Comments

There are a number of disadvantages to current pyrogen-detection methods. These have been discussed previously, but necessitate a brief mention. The RPT exposes live rabbits to painful or distressing experiences; requires trans-species extrapolation; is less sensitive than the human fever threshold;[2] and is ill equipped to handle substances such as cellular products, radiopharmaceuticals, certain biologicals, and medical devices. The *LAL* also requires species extrapolation, can only detect LPS, and cannot be used for substances that interfere with the clotting process, biologicals ,or the direct assessment of medical devices.

Despite references to the 3Rs, the RPT is still used extensively, especially for complex biologically derived products and end-product release testing. Indeed, it is estimated that up to 400,000 rabbits per year are used,[3] and the LAL, despite catch-and-release procedures, results in an approximate 15% mortality rate.[4] It is therefore imperative, for both ethical and scientific reasons, that both of these tests are replaced by the alternatives presented here for endorsement.

In addition to the obvious ethical advantages of human whole and/or cellular blood pyrogenicity tests, the IVPTs have numerous scientific advantages. The first is the elimination of species extrapolation issues, since the proposed test methods are direct *in vitro* models of the human fever response. Additionally, because the pyrogenic response is a blood-mediated reaction, IVPTs are not limited by potential *in vivo/in vitro* extrapolation considerations, as some *in vitro* tests might be. The IVPTs are sensitive and can detect all potential pyrogens, not only LPS. They can be used to evaluate traditional pharmaceuticals as well as medical devices, species-specific cellular/biological therapies, cell culture media, air quality assessments, and human serum albumin, among other materials. The IVPTs could also be easily adapted into species-specific pyrogenicity tests for veterinary products.

The methods presented to the panel have undergone a full quantitative validation study. The validation studies were conducted in order to certify the IVPTs as appropriate for replacement of both the RPT and the LAL. The concordances and sensitivities for all five human blood-based methods are over 90%; specificities are above 80%; and all methods demonstrate low false-positive and -negative rates.[5] In comparison, historical data from 171 rabbits were used to calculate a theoretical sensitivity of 57.9% and a theoretical specificity of 88.3% for the RPT.[4]

Clearly, the IVPT methods, after 20 years of research and refinement, are a wholly superior way to detect pyrogens in medicinal products. However, the animal protection community has serious concerns related to the duplication of review efforts, as evidenced by the time ICCVAM has taken to arrive at this point with the IVPTs. As discussed in another recent set of public comments, ICCVAM continues to invest substantial time and resources in what are regarded by many as redundant and unnecessarily duplicative evaluations of 3Rs methods that have already undergone successful validation, independent peer review, and/or international acceptance in other jurisdictions. We therefore question the value of subjecting the IVPTs to multiple peer reviews—particularly when the animal-based RPT and LAL have not been subject to a level of scrutiny even closely resembling that of an ECVAM or ICCVAM validation study.

Specific Recommendations

Accept IVPTs as full replacements for the LAL

It is unclear why ICCVAM has chosen not to consider the IVPTs as appropriate for replacement of both the RPT and the LAL. With the validation of the IVPTs using an endotoxin standard, the LAL has become redundant. If there are specific cases of which we are not aware that require the LAL, exceptions can be made, but surely for ethical and scientific reasons the IVPTs should in general replace the LAL.

Certify the IVPTs valid for the detection of all pyrogens; conduct a "retrospective validation," if needed.

The mechanism of action behind the detection of LPS in the LAL, and hence the reason for its pyrogen specificity, is unique to arthropods. The mechanism of action, if not the magnitude of response, behind the detection of pyrogens in the RPT and the IVPTs is the same. Since the RPT is currently used to detect all pyrogens, there is no biologically sound rationale to conclude that the IVPTs cannot also detect all pyrogens—at a level at least equivalent to the RPT. ICCVAM documents drafted for review today state as much.

Indeed, BRDs submitted by ECVAM, draft BRDs posted by ICCVAM, and other materials list between 15 and 30 published studies discussing the detection of pyrogens, including non-LPS pyrogens, in human serum albumin, pharmaceuticals, and other materials. Some studies used clinically positive materials, and some used comparisons to the traditional *in vivo* or an *in vitro* version of the RPT.[6-8] One of these studies[6] compared the WB/IL-1 IVPT and the RPT using 96 batches of parenteral pharmaceuticals. Of all test substances, only one tested positive in all three (RPT, LAL, and WB/IL-1) test systems. The remaining 95 were negative in all test systems. ECVAM has also provided detailed testing results of materials with the IVPT methods that were determined to be positive for pyrogenic activity during clinical experience. Results were favorable in all assessments.[4]

It is at best perplexing to see peer review reports and testing recommendations stop short of giving the IVPT methods full validated certification, and only recommend the use of these methods for the detection of LPS-mediated pyrogenicity. While most pyrogenicity is indeed related to LPS, the ICCVAM draft recommended test method uses and future studies virtually guarantee that the RPT will not be replaced in the foreseeable future, as it will be needed to certify regulated end products completely "pyrogen free."

Given the results of Jahnke[6] above, it is further difficult to envision the concurrent *in vivo/in vitro* study recommended by ICCVAM. Hundreds of rabbits could be used in an unnecessary quest to get enough non-LPS-mediated pyrogenicity reactions in rabbits to subsequently confirm using the IVPT methods.

For ethical reasons, the ECVAM validation did not include such concurrent testing. Instead, the study chose LPS, a model pyrogen, to represent the pyrogen reaction and validate the *in vitro* test systems. There is no scientific reason to suspect that the IVPTs will not detect the full range of pyrogens. Published evidence supports this hypothesis,[7-10] as does supporting evidence submitted by ECVAM in early 2006.[4] If necessary, a coordinated assessment of such evidence—a retrospective validation of sorts—should more than allay any concerns about the applicability of the IVPTs to all varieties of pyrogen.

Articulate more clearly a path to full replacement

Investments in IVPTs by industry and the public sector are increasing. At least one American company, Charles River Laboratories, has for some time offered an IVPT assay for use in the detection of the range of pyrogens for research use. At least 200 laboratories worldwide have worked with or offer similar assays. Faith in the continued growth of these methods is clearly held by industry, academia, and government alike. With approval and continued use, we are confident that the IVPT methods will become

the "Gold Standard" for human pyrogen detection. The ICCVAM recommendations as currently written will limit the usefulness of these assays, and fail to achieve real reductions in animal use in a timely manner. We urge ICCVAM to revise its Recommendations as outlined above—and offer detailed guidance on how prospective end-users can adopt the IVPTs and put them into immediate practice.

Thank you for your attention to these comments.

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